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Review

The *ALMT* Gene Family Performs Multiple Functions in Plants

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Abstract: The *aluminium activated malate transporter* (*ALMT*) gene family is named after the first member of the family identified in wheat (*Triticum aestivum* L.). The product of this gene controls resistance to aluminium (Al) toxicity. *ALMT* genes encode transmembrane proteins that function as anion channels and perform multiple functions involving the transport of organic anions (e.g., carboxylates) and inorganic anions in cells. They share a PF11744 domain and are classified in the Fusaric acid resistance protein-like superfamily, CL0307. The proteins typically have five to seven transmembrane regions in the N-terminal half and a long hydrophillic C-terminal tail but predictions of secondary structure vary. Although widely spread in plants, relatively little information is available on the roles performed by other members of this family. In this review, we summarized functions of *ALMT* gene families, including Al resistance, stomatal function, mineral nutrition, microbe interactions, fruit acidity, light response and seed development.

Keywords: *ALMT* gene family; anion channels; aluminium resistance; stomatal function

1. Introduction

The *aluminium activated malate transporter* (*ALMT*) gene family is named after the first member of the family identified in wheat (*Triticum aestivum* L.) [1]. The product of this gene controls resistance to aluminium (Al) toxicity. *TaALMT1* was the first member of this novel family of genes encoding anion channels. Although widely spread in plants, relatively little information is available on the roles performed by other members of this family. However those that have been characterised show a range of functions including Al resistance [1–4], stomatal function [5–11], mineral nutrition [12,13], fruit acidity [14,15], microbe interactions [16,17] and seed development [18].

ALMT genes encode transmembrane proteins that function as anion channels and perform multiple functions involving the transport of organic anions (e.g., carboxylates) and inorganic anions in cells [19–21]. Carboxylic acids such as malic and citric acids are important metabolites in plant cells due to their involvement in photosynthesis, the tricaboxylic acid pathway, carbohydrate metabolism, cytosolic pH regulation, contribution to the maintenance of electroneutrality and nutrition [22–24]. These organic anions also confer resistance to Al toxicity in acid soils because when released from roots their carboxyl groups chelate the trivalent Al cations (Al^{3+}) rendering them less toxic [22]. Carboxylic anions can also bind with other multivalent metal ions [21].

Anion channels are integral membrane proteins that form aqueous pores to selectively allow the rapid transport of anions across membranes down their electrochemical gradients [25]. Therefore, anion channels are passive transporters located on many plant membranes including the plasma membrane, tonoplast, endoplasmic reticulum, mitochondrial and chloroplast membranes. Some also perform signalling roles and function as master switches in stress responses [25–27]. The

development of the patch-clamping technique provided a method for detailed research on the functioning of ion channels. The patch-clamp technique has been used to investigate the function of some members of the ALMT family. Some are permeable to malate anions while others are also permeable to other organic inions (such as fumarate) or inorganic inions (such as Cl^-, NO^{3-} and less to SO_4^{2-}). Other useful methods for studying ion channels also include the analysis of substrate concentrations in native and heterologous expression systems. In plants, the guard cells that regulate stomatal aperture have become a popular model system for characterising membrane transport and signal transduction. For instance, in *Vicia faba* L. guard cells, researchers have identified two types of anion channel considered important for stomatal closure: the rapid-type (R-type) and the slow-type (S-type) [28]. The R-type anion channels can be activated rapidly within 50 ms by depolarisation and activates potassium release when the membrane potential falls below the equilibrium potential for K^+ while S-type channels showed a much slower (seconds) voltage-dependent activation and deactivation [20,28].

2. *ALMT* Genes Perform Various Functions among Plants

In acid soils the soluble Al ions exist mainly in the trivalent (Al^{3+}) form which damages membranes and rapidly inhibits root growth [29]. A widespread mechanism for Al resistance in many plant species relies on the release of organic anions from roots apices (the sensitive region of the root for Al toxicity). The organic anions, such as malate, citrate and oxalate, are able to chelate Al^{3+} to prevent it from binding in the cell wall and membranes and from entering the cytosol. This chelation reduces harmful interactions from occurring in the apoplast and in the cytosol of the root apex [30]. The first member of the family identified was *TaALMT1* from wheat where it controls the major mechanism for aluminium (Al^{3+}) resistance [1]. *TaALMT1* is constitutively expressed in the root apices and activated by the Al^{3+} cations prevalent in acid soils to release malate anions into the apoplast. Malate chelates the Al^{3+} which reduces damage to the cell wall, membranes and other cellular components. Other members of the family perform similar functions in *Arabidopsis* [3,31], rape (*Brassica napus* L.) [4], rye (*Secale cereale* L.) [2], soybean (*Glycine max* L.) [32], alfalfa (*Medicago sativa* L.) [33], and Yorkshire fog (*Holcus lanatus* L.) [34]. Regulation of these vary and some show more complex interactions [35]. For instance, *AtALMT1* is not constitutively expressed in *Arabidopsis* roots but induced by low pH and Al^{3+} [3] and *GmALMT1* in soybean shows complex interactions with Al stress and phosphorus nutrition [32].

Most members of the ALMT family are not involved in Al^{3+} resistance but perform other functions on different membranes [21,36]. For example, four members in *Arabidopsis* (*AtALMT4*, *AtALMT6*, *AtALMT9* and *AtALMT12*) and one in barley (*Hordeum vulgare* L., *HvALMT1*) are involved in guard cell regulation. This is consistent with the involvement of anion transport in signal transduction and osmotic adjustment in modulating stomatal aperture [5–7,10,18,20]. Electrophysiological studies in *Xenopus laevis* (Daudin, 1802) oocytes revealed similarities between the currents generated by *AtALMT12* and the previously characterised rapid or R-type/QUAC anion currents which propose that *AtALMT12* anion channels are responsible for these currents [6,37]. *AtALMT6* localises to the tonoplast of guard cells and facilitates calcium and pH-dependent movement of malate into the vacuole [8], but knockout mutations show little or no phenotype indicating some redundancy in these functions. *AtALMT9* also localises to the tonoplast of guard cells and knock-out mutations show impaired stomatal opening in the light and delayed wilting [8,9]. *AtALMT9* is activated by cytosolic malate and appears to facilitate chloride movement into the vacuole during stomatal opening. *VvALMT9* is a homolog of *AtALMT9* in grape (*Vitis vinifera* L.) and reportedly controls the acidity of berries during fruit maturation by facilitating tartrate, malate and succinate accumulation in the vacuoles of mesocarp cells [15]. A recent study also indicated that the ABA induced anion channel AtALMT4 of mediates Mal^{2-} efflux from the vacuole during stomatal closure and its activity depends on phosphorylation [38].

Acidity in apple (*Malus domestica* Borkh.) similarly relies on the ALMT transporter Ma1 which mediates malate accumulation into the vacuoles of developing fruit cells [14]. In maize (*Zea mays* L.), *ZmALMT1* and *ZmALMT2* are mainly expressed in root tissues and likely function to assist in

balancing charges during nutrient uptake [12,13]. Both proteins localise to the plasma membrane and both are permeable to inorganic anions but *ZmALMT2* is also permeable to organic anions as demonstrated by the constitutive release of malate from the roots of transgenic *Arabidopsis* plants expressing *ZmALMT2* [13]. *OsALMT4* is a member of the *ALMT* family in rice (*Oryza sativa* L.). *OsALMT4* is widely expressed in rice and encodes a malate-permeable anion channel on the plasma membrane. Enhancing *OsALMT4* expression affected the compartmentation of malate within the tissues which increased Mn concentrations in the apoplast and caused the toxicity symptoms of dark brown spots and stripes and occasionally necrotic margins [39].

3. ALMTs Are Widely Distributed in the Genomes of Higher Plants

The characterised ALMT members show a range of functions including Al resistance [1–4], stomatal function [5–11], mineral nutrition [12,13,39], microbe interactions [16,17], fruit acidity [14,15], and seed development [18]. Members which have been characterised are widely distributed in higher plant families of *Poaceae*, *Brassicaceae*, *Fabaceae*, *Rutaceae, Rosaceae*, *Vitaceae* and *Solanaceas* (Table 1).

Table 1. Functions and characteristics of members of the aluminium activated malate transporter (ALMT) family.

Family	Species	Gene	Function(s)
Poaceae	Wheat	*TaALMT1*	Al Resistance
	Rye	*ScALMT1*	Al Resistance
	Goatgrass	*AetALMT1*	Al Resistance
	Barley	*HvALMT1*	Stomatal Functions, Seed development
	Yorkshire fog	*HlALMT1*	Al Resistance
	Maize	*ZmALMT1*	Mineral Nutrition
		ZmALMT2	Mineral Nutrition
	Rice	*OsALMT4*	Mineral Nutrition
Brassicaceae	*Arabidopsis*	*AtALMT1*	Al Resistance, Microbe interactions
		AtALMT4	Stomatal Functions
		AtALMT12	Stomatal Functions
		AtALMT6	Stomatal Functions
		AtALMT9	Stomatal Functions
	Rape	*BnALMT1*	Al Resistance
		BnALMT2	Al Resistance
Fabaceae	Alfalfa	*MsALMT1*	Al Resistance
	Soybean	*GmALMT1*	Al Resistance
	Lotus japonicus L.	*LjALMT4*	Microbe interactions
Rutaceae	Citrus	*CsALMT1*/*CgALMT1*	Al Resistance
Rosaceae	Apple	*Ma1*/*Ma2*	Fruit acidity
Vitaceae	Grape	*VvALMT9*	Fruit acidity
Solanaceas	Tomato	*SlALMT4*	Unclear
		SlALMT5	Seed malate content
		SlALMT9	fruit malate accumulation, Al Resistance

3.1. ALMT Members in Poaceae

The Poaceae species which have characterised ALMT members include wheat TaALMT1, rye ScALMT1, goatgrass AetALMT1, Yorkshire fog HlALMT1, maize ZmALMT1 and ZmALMT2, rice OsALMT4 and barley HvALMT1. ALMT genes in wheat, rye, goatgrass and Yorkshire fog facilitate

resistance to Al, members in maize and rice functions on mineral nutrition, while the barley HvALMT1 was related to stomatal functions (Figure 1).

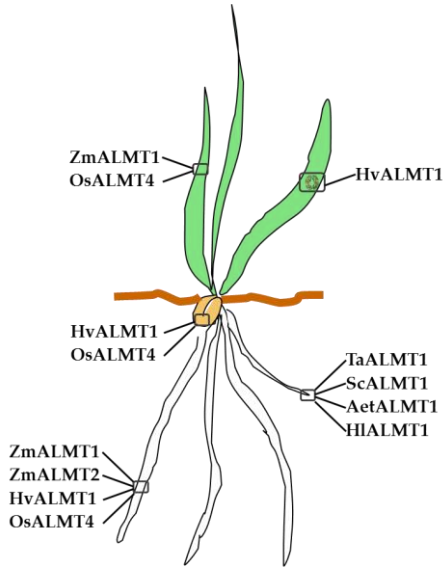

Figure 1. ALMT members within *Poaceae*. Within *Poaceae*, *TaALMT1*, *ScALMT1*, *AetALMT1* and *HlALMT1* are expressed in root tips; *ZmALMT1* and *ZmALMT2* are expressed in mature root while *ZmALMT1* also has expression in leaf; *OsALMT4* is widely expressed in the rice plant but highly in vasculature, and *HvALMT1* expressed in mature root, seed and guard cells.

By using a cDNA subtractive hybridisation technique, Sasaki et al. [1] cloned a cDNA which was more highly expressed in the root apices of an Al-resistant wheat line (ET8) than in a near-isogenic but Al-sensitive line (ES8). *TaALMT1* was mapped to chromosome 4DL and the relative expression of this gene among various genotypes of wheat was strongly correlated with Al-activated malate efflux capacity and Al resistance [40]. Electrophysiological characterisation in *Xenopus laevis* oocytes indicated that *TaALMT1* encodes an Al-activated transporter that can mediate both inward and outward currents. The malate transport permeability is enhanced by external Al but it is also permeable to a lesser degree to other physiologically relevant anions such as Cl^- and NO_3^- [1,41]. Transgenic rice, *Arabidopsis*, barley and tobacco cultured cell lines expressing *TaALMT1* all showed an Al-activated malate efflux. In the later three cases, *TaALMT1* also increased Al resistance [1,42]. The higher expression of *TaALMT1* in resistant than sensitive wheat genotypes is due to a series of repeated blocks in the promoter [43].

Among cereal crops, rye is the most Al resistant species. Researchers have mapped Al-resistance loci on chromosomes 3R (*Alt2*), 4RL (*Alt3*), 6RS (*Alt1*) and 7RS (*Alt4*) by using segregating populations and wheat-rye addition lines [44–48]. Using primers to the *TaALMT1* gene, Fontecha et al. [49] cloned a 1359 bp cDNA called *ScALMT1* from rye and showed that it co-segregated with the Al resistance locus *Alt4*. *ScALMT1* is mainly expressed in roots and expression is upregulated by exogenous Al treatment. Collins et al. [2] showed that the *Alt4* resistance locus contains a cluster of *ALMT* genes. Although there are some mRNA splice variations that might affect protein function, the *M77.1* promoter and *ScALMT1-M39.1* gene are sufficient to explain most of the Al resistance in that line. As the ancestor of the wheat D genome, goatgrass (*Aegilops tauschii* Coss.) contains more genetic variation than bread wheat perhaps because it experienced less artificial selection. Ryan et al. [50] screened 760 accessions in a total of seven progenitor species of hexaploid wheat and other early relatives. Of these only five of 29 *Aegilops tauschii* tested showed a moderate level of resistance to Al. The coding sequences of the *AetALMT1* genes in the five resistant genotypes were identical to *TaALMT1* and resistance was correlated with *AetALMT1* expression level and with Al-activated malate efflux. Unlike *TaALMT1*, all the *Aegilops tauschii* genes lacked any of the tandem repeats in the promoter region—even the moderately resistant ones [50]. Yorkshire fog is a grass that shows a large genotypic variation in acid soil tolerance. Chen et al. [34] cloned the *HlALMT1* gene and found that

plants from plots with acidic soil (HL-A) had significantly higher *HlALMT1* expression levels in their roots than plants from plots with neutral pH soil (HL-N). The higher expression in HL-A plants was linked with a greater number of the cis-acting elements which recognise an Al-responsive transcription factor (HlART1) [34]. Furthermore, the HL-A roots secreted approximately twice as much malate as the HL-N roots, but there was no difference in citrate secretion. These results indicated that cis elements in the promoter of the *HvALMT1* gene generated higher expression of *HlALMT1* leading to higher malate efflux and greater Al resistance.

In maize, *ZmALMT1* was initially considered a candidate for an Al resistance gene due to its sequence homology with *TaALMT1* [12]. Although *ZmALMT1* localises to the plasma membrane, is mainly expressed in root tissue, and shows increased expression with Al treatment, other results indicated that this gene was not involved with the mechanism of Al resistance. Firstly, electrophysiological characterisation in *Xenopus* oocytes showed that ZmALMT1 had greater permeability to NO_3^- and Cl^- than malate and these anions would not bind with Al^{3+} to reduce its activity in the apoplast. Furthermore, *ZmALMT1* expression was low in the root tips where protection from Al^{3+} stress by organic anions is crucial. Lastly, transport activity of *ZmALMT1* was independent of exogenous Al [12]. Linkage analysis of Al resistance genes in maize identified another *ALMT* gene, *ZmALMT2*, that is associated with net root growth under Al stress [51]. However, once again, *ZmALMT2* is more permeable to inorganic anions (NO_3^-, SO_4^{2-} and Cl^-) than organic anions such as malate. Furthermore, *ZmALMT2* is mainly expressed in mature roots and not root apices, expression levels are similar in Al-sensitive and resistant genotypes. Therefore, this gene is also unlikely to be involved with Al resistance despite its genetic linkage with that trait. Instead the authors suggested *ZmALMT2* is involved with mineral nutrition [13].

There are nine members of the ALMT family rice but only *OsALMT4* has been examined in detail. *OsALMT4* localises to the plasma membrane and is expressed widely in roots and shoots, especially in the vasculature. Transgenic plants over-expressing *OsALMT4* released malate from roots which is consistent with *OsALMT4* encoding a malate-permeable anion channel. The over-expressing (OX) lines developed distinct symptoms on their leaves which consisted of dark brown spots and stripes and occasionally necrotic margins which did not appear on null segregant plants or RNAi lines with reduced *OsALMT4* expression. The partitioning of Mn within the leaf tissue was altered in the transgenic since the OX lines had a greater proportion of Mn residing in the apoplast of the leaves compared to the null plants. OX lines also had higher concentrations of malate in the xylem sap and increased expression of genes encoding Mn transporters (*OsNramp5* and *OsYSL2*) and B transporters (*OsBOR1* and *OsNIP3.1*). Growth studies found that the OX lines were more sensitive to Mn toxicity but more tolerant of B toxicity than null plants. The authors conclude that enhancing *OsALMT4* expression affected the compartmentation of malate within the tissues which increased Mn concentrations in the apoplast and caused the toxicity symptoms [39].

Barley is another important plant in *Triticeae* and the barley *ALMT1* gene was shown to be involved in facilitating anion efflux from guard cells during stomatal closure and seed development [5,11,18]. *HvALMT1* is located on chromosome 2H. Tissue localisation showed that this protein was expressed in guard cells and in emerging lateral roots, the nucellar projection, aleurone layer and the scutellum of developing barley grain [5,18]. Intracellular localisation of *HvALMT1* included the plasma membrane and small motile vesicles in the cytosol but the identity and function of those vesicles is unknown. Heterologous expression in *Xenopus* oocytes showed that this protein could mediate both inward and outward currents. *HvALMT1* is permeable to malate and its activity is sensitive to pH [5]. The transgenic plants over-expressing *HvALMT1* released more malate from roots than the control plants and they took significantly longer to close their stomata under low light than null controls. They also tended to reach a lower stomatal conductance under low light but the results were not significant. Combined with the finding that *HvALMT1* is highly expressed in guard cells these results provided evidence that this channel contributes to stomatal function [11]. Interestingly, overexpressing *HvALMT1* to high levels with a constitutive promoter affected plant growth and grain formation. Further experiments showed that malate release measured from isolated aleurone layers of grain was significantly lower in transgenic lines in which *HvALMT1* expression was reduced by

RNAi compared to control plants [18]. Therefore, *HvALMT1* function affects stomatal aperture, grain development and plant growth. This shows that a single *ALMT* gene may perform multiple functions [5,11,18].

3.2. ALMT Members in Brassicaceae

The *Arabidopsis ALMT* gene family contains 14 members and five of them were detailed characterised (Figure 2). *AtALMT1* is mostly expressed in Arabidopsis roots and knock-out mutants are more sensitive to Al stress [3]. Expression studies showed that *AtALMT1* expression is induced by Al treatment and low pH [3]. Electrophysiological characterisation in *Xenopus* oocytes indicated that *AtALMT1* functions as a malate-permeable channel [3,52]. However other researchers could not demonstrate *AtALMT1* function in oocytes [53]. Interestingly, *AtALMT1* expression is not only induced by external Al but also by low pH, hydrogen peroxide and phytohormones such as ABA and IAA [54]. Furthermore, *AtALMT1* might also be involved in biotic interactions. A transgenic *Arabidopsis* line in which GUS expression was driven by the promoter of *AtALMT1* showed an increase in GUS expression in the roots when the leaves were treated with the foliar pathogen *Pseudomonas syringae* pv *tomato* (Pst DC3000). This same treatment also induced malate release from the roots. The authors found that the malate release helped recruit the beneficial bacterium *Bacillus* FB17 around the roots [16,31]. Furthermore, *AtALMT1* expression was induced by *flg22*, a kind of MAMP (microbe-associated molecular pattern) in a manner that was independent of the salicylic acid and jasmonic acid pathways [55]. A recent study with *Arabidopsis*, which also relies on malate efflux via a *ALMT1* channel, proposes that the Al:malate complex is taken up by the root cells and transported to shoot by NIP1;2 to maintain low Al concentrations in the apoplast shoots [56]. These results indicate that *AtALMT1* expression could contribute to plant-pathogen interactions [16] and that a single *ALMT* gene can perform multiple functions.

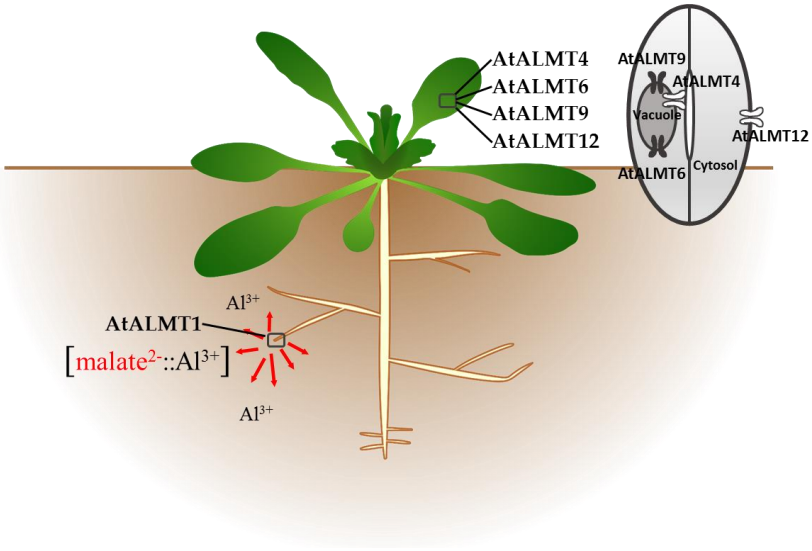

Figure 2. ALMT members in *Arabidopsis*. *AtALMT1* is expressed in root and performs the function of Al resistance by excreting malate. *AtALMT6*, *AtALMT9* and *AtALMT12* are expressed in leaf guard cells. *AtALMT4* and 12 is involved in regulating stomatal closure while *AtALMT6* and 9 function during stomatal opening.

The *Arabidopsis ALMT12* gene was the first member of the family to be implicated in stomatal function. Mutant analysis demonstrated that *AtALMT12* is involved in regulating stomatal closure but not opening. *AtALMT12* is predominantly expressed in guard cells. The expression level of *AtALMT12* in shoots is 10 times higher than that in roots and it is mainly expressed in guard cells [7]. Electrophysiological studies of guard cells during stomatal closure divided the inward currents (which by convention involves anion efflux) into those mediated by a rapid anion channel (R-type) also known as quick-activating anion channels (QUAC), and a slow anion channels (S-type) also

known as the slow-activating anion channels (SLAC). The R-type anion channels can be activated rapidly within 50 ms by depolarisation and activates potassium release when the membrane potential falls below the equilibrium potential for K^+ while S-type channels showed a much slower (seconds) voltage-dependent activation and deactivation [20,28]. Initial reports disagreed as to the sub-cellular localisation and function of *AtALMT12*. Sasaki et al. [7] localised this protein to endomembranes and plasma membrane and predicted that *AtALMT12* can mediate Cl^- and NO_3^- fluxes but not malate. They also concluded that this protein was not responsible for either the R-type not S-type anion currents [7]. By contrast, Meyer et al. [6] found that the protein localised to the plasma membrane and concluded that *AtALMT12* can mediate malate transport and likely functions as the R-type anion channel. Further studies confirmed that *AtALMT12* is a malate-dependent R-type channel in guard cells and interacts with the kinase Open Stomata 1 to regulate stomatal closure [6,37,57]. Similarly, a recent report mentions that the *ALMT4* is required for stomatal closure in response to abscisic acid (ABA). *AtALMT4* functions as an ion channel that mediates Mal^{2-} release from the vacuole. *ALMT4* is localised to the vacuolar membrane and is expressed in leaf mesophyll and guard cells. Electrophysiological result indicates that *ALMT4* can mediate Mal^{2-} efflux and the channel activity is dependent on a phosphorylatable C-terminal serine. Knock-out mutants showed impaired stomatal closure in response to the drought-stress hormone ABA and increased whole-plant wilting in response to drought and ABA [38].

Malate movement can also be important to balance the accumulation of cations in the vacuole such as potassium during stomatal opening. Therefore, *ALMTs* probably contribute to balancing anion movement across the tonoplast as well. For instance, in *Arabidopsis* the AtALMT6 and AtALMT9 proteins localise to the tonoplast. *AtALMT6* is expressed in guard cells of leaf, stem and flower tissues. An AtALMT6-GFP fusion protein was targeted to the vacuolar membrane both in transient and stable expression systems [8]. Meyer et al. [8] concluded that *AtALMT6* is a Ca^{2+}-activated malate channel as its transport activity was significantly altered by cytosolic Ca^{2+}. Moreover, *AtALMT6* activity can be regulated by vacuolar pH and cytosolic malate. The *atalmt6* T-DNA knockout mutant showed reduced malate current density across the tonoplast membrane [8]. Kovermann et al. [10] cloned the *AtALMT9* gene and 1785 bp upstream of the coding region. Subcellular localisation in both *Arabidopsis* and onion epidermal tissue showed localisation to the tonoplast. Tissue-specific localisation using the promoter to drive *GUS* expression showed the gene is expressed in many organs including leaves (mesophyll tissue), roots and flower tissues [10]. Malate currents were inhibited across the tonoplast of *AtALMT9* knock-out lines compared to WT controls but enhanced in tobacco lines expressing *AtALMT9*. *AtALMT9* appears to function primarily as a malate channel although it exhibits weak conductance to fumarate and chloride [10]. More recently, De Angeli et al. [9] confirmed that *AtALMT9* is an anion channel located on the tonoplast and is involved with stomatal opening. Unlike *AtALMT6*, *AtALMT9* is not sensitive to cytosolic Ca^{2+} but can be activated by malate in the cytosol but not by malate in the vacuole [9]. Further research also demonstrated that *AtALMT9* transport can be modulated by cytosolic nucleotides and other vacuolar anions [58].

Unlike other plant species, rape releases both malate and citrate when exposed to Al [59]. Ligaba et al. [4] cloned two *TaALMT1* homologs from rape. These proteins only have 40% similarity with the wheat gene, but they still proved to be related to Al resistance. Similar with other Al related members, *BnALMT1* is localised in the plasma membrane [4]. The expression of *BnALMT1* and *BnALMT2* was detected mainly in root but not shoots and their expression can be enhanced by Al treatment and to a lesser degree by other multivalent cations (such as lanthanum, ytterbium, and erbium). Transgenic tobacco culture cells expressing these genes showed increased malate efflux when exposed to Al and some other cations such as ytterbium and erbium [4].

3.3. ALMT Members in Fabaceae

Medicago sativa (lucerne or alfalfa) is an Al-sensitive pasture legume species. Nevertheless transcript analysis using a cDNA library from cultivar YM1 SSH indicated that *MsALMT1* expression was up-regulated by a 24 h treatment with 5 μM Al [60]. Transgenic tobacco expressing *MsALMT1*

showed malate efflux and the rate correlated with *MsALMT1* transcription level. Under Al stress, these transgenic tobacco plants also had better root growth than control lines which suggests that this gene could be involved with Al resistance in *Medicago* even though it was cloned from a sensitive genotype [33].

GmALMT1 from soybean has been cloned by Liang et al. [32]. Sub-cellular localisation of the protein indicated that it is located in the plasma membrane. Heterologous expression in *Xenopus laevis* cells shows that this gene encodes a protein that mediates inward and outward currents. *GmALMT1* is mainly expressed on root tips with expression and malate efflux altered by both low pH and Al treatments. *Arabidopsis* plants overexpressing *GmALMT1* show better malate efflux and higher Al resistance. Additionally, increasing and decreasing the expression of this gene in transgenic hairy roots of soybean indicated that accumulation of Al in the root tissues was inversely correlated with relative *GmALMT1* expression level. Together these results demonstrate that *GmALMT1* is also likely to function as an Al resistance gene in soybean [32].

Of the seven ALMT proteins expressed in *Lotus japonicus LjALMT4* is highly expressed in nodules. When expressed in *Xenopus laevis* oocytes, this protein was found to mediate the efflux of dicarboxylates, including malate, succinate, and fumarate, but not tricarboxylates such as citrate. Moreover, *LjALMT4* was found to be specifically expressed in the parenchyma cells of nodule vascular bundles, suggesting that *LjALMT4* is not a transporter at the peribacteroid membrane but is involved in transporting [17].

3.4. ALMT Members in Fruit Plants

In many acid soils it is common for Al toxicity to be linked with phosphorus (P) deficiency. This is because the soluble P is likely to be in a complex with iron or Al or fixed in other less soluble forms. Indeed some plants very efficient at taking up P from highly P-fixing soils, such white lupin (*Lupinus albus* L.), rely on organic anion release from roots (especially citrate release) to access more P [22]. Research indicates that members of the ALMT family might also contribute to greater P-use efficiency. For instance, the Al resistant species of citrus 'Xuegan' (*Citrus sinensis* (L.) Osbeck, *CsALMT1*) displayed more resistance to P-deficiency than a sensitive species 'Sour pummelo' (*Citrus grandis* (L.) Osbeck, *CgALMT1*). Evidence was presented that *ALMT* genes in these species might contribute to the resistance of citrus to Al and/or P-deficiency [61].

Some ALMT proteins appear to affect fruit acidity by transporting malate across the tonoplast membrane (Figure 3). This was revealed when Bai et al. [14] narrowed down the *Ma* locus to a 65–82 kb region on chromosome 16 of the apple cultivar Golden Delicious. The *Ma* locus is associated with fruit acidity in apple and the genomic region contains 12–19 predicted genes. By aligning these genes with two BACs covering different haplotypes of the *Ma* locus (high and low acidity), they identified two *ALMT* homologues and named them *Ma1* and *Ma2*. Phylogenetic analysis using these two apple ALMT members against the Arabidopsis proteins indicated that these two members from apple were most similar to *AtALMT6* and *AtALMT9* which mediate anion transport across the tonoplast [14]. *Ma1* was shown to localise on the tonoplast and heterologous expression in yeast increased malate influx into yeast cells. Quantitative RT-PCR experiments predicted that expression of *Ma1*, but not *Ma2*, is significantly linked with fruit acidity (high *Ma1* expression was correlated with more acidic fruit). Moreover, a natural mutation in *Ma1* leading to a truncation of the gene was also associated with low fruit acidity. The *Ma1* genotype is closely related to apple fruit acidity suggesting *Ma1* is associated with the accumulation of malic acid in apple fruits [62]. Similarly, De Angeli et al. [15] cloned an *AtALMT9* homolog from grape called *VvALMT9*. *VvALMT9* expression increased as the berry matured. The VvALMT9 protein has 64% identity with AtALMT9 and also localises to the tonoplast. *VvALMT9* is not only permeable to malate, but also displays a low permeability to tartrate. The authors predicted that *VvALMT9* is a tonoplast channel that contributes to malate and tartrate accumulation in grape berries [15].

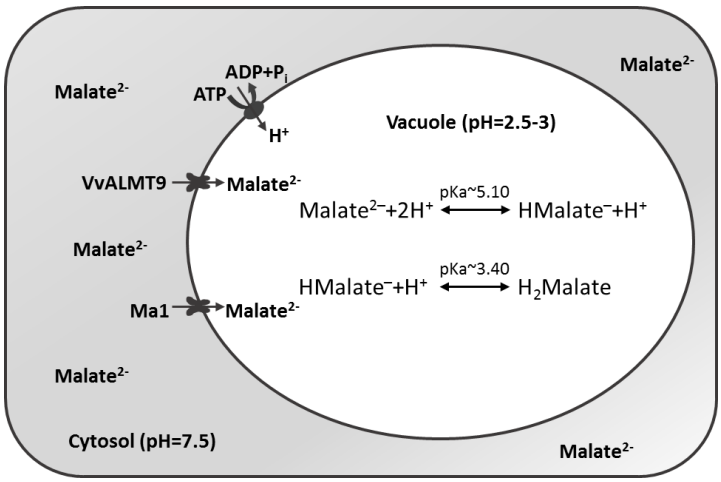

Figure 3. ALMTs contributing to fruit and berry acidity. The pH of the cytosol is neutral or slightly alkaline so malate exists mainly in the malate^{2-} form. In the vacuole, where the pH is acidic, a larger proportion of malate exists either as the protonated malic acid or the mono-valent anion (malate^{-}). Once malate^{2-} is transported from the cytosol to the vacuole across the tonoplast, a proportion will be protonated which partly helps to maintain the electrochemical potential gradient for malate^{2-} influx into the vacuole [63]. Also shown is an H^+ pump which transports H^+ into the vacuole. This figure is modified from De Angeli et al. [15].

As a model system for fruit research, the function of some ALMT members in tomato (*Solanum lycopersicum* L.) also examined in detailed. *SlALMT4* and *SlALMT5* are expressed in fruit, leaf, roots and flowers, *SlALMT6* and *SlALMT9* only in flowers, and *SlALMT7* in flowers, roots and immature fruit [64]. Both *SlALMT4* and *SlALMT5* are expressed in the endoplasmic reticulum and *SlALMT5* is also expressed on endomembranes. The *SlALMT9* was predicted to localise on vacuole membrane [65]. Both *SlALMT4* and *SlALMT5* have the ability to transport malate under electrophysiological system, but *SlALMT5* is also permeable to inorganic anions such as nitrate and chloride. *SlALMT5* overexpressed tomato exhibited higher malate and citrate concentrations in mature seed which indicated the function of *SlALMT5* on mature seed [65]. No detailed information was reported on the function of *SlALMT4*. By using GWAS and linkage mapping, *SlALMT9* was found to be a causal candidate gene for the natural variation of tomato fruit malate content [65]. This variation is closely related to the 3-bp indel in the promoter region of *SlALMT9*, which affects the W-box binding site in the *SlALMT9* promoter and the ability of binding the WRKY transcription repressor Sl-WRKY42. *SlALMT9* also confers the function of Al resistance [65].

4. Structural Analysis and Evolution of the ALMT Family

4.1. Phylogeny of the ALMT Family

Identification of *TaALMT1* in wheat stimulated further interest in this novel family of genes as described above. When Delhaize et al. [19] analysed the phylogeny of the ALMT family they used the UPF0005 (uncharacterised protein family five domain) for identifying other members. Using UPF0005 as the search sequence they concluded that the ALMT family was restricted to the seed-forming plants (Spermatophyta) [19]. The possible inclusion of ALMT in other phylogenetic groups other than the plants was largely unexplored. With the continual update of the Pfam database, the UPF0005 domain has been renamed to Bax1-I (Inhibitor of apoptosis-promoting Bax1, PF01027, http://pfam.xfam.org/family/pf01027) which is a member of the Apoptosis-Inhib Clan (CL0453, http://pfam.xfam.org/clan/cl0453). The ALMTs have been removed from that group to its own category (aluminium activated malate transporter, PF11744, http://pfam.xfam.org/family/PF11744.7) which falls within the FUSC Clan (Fusaric acid resistance protein-like superfamily, CL0307, http://pfam.xfam.org/clan/FUSC) [66]. The PF11744 domain contains the WEP fingerprint motif (Trp-Glu-Pro) present in all ALMTs [20]. Until recently, the ALMT family has been generally regarded as

specific to plant species [67] and largely angiosperms. Dreyer et al. showed that the distribution of ALMTs included the Bryophyta (mosses) and Lycophyta (vascular moss or club moss, e.g., *Selaginella* sp.) [9,19–21]. Before the first ALMT member was identified, Harley and Saier defined a PET (Putative Efflux Transporter) family which included efflux transporters and proteins from bacteria, plants, yeast and protozoans [68]. By aligning the plant proteins in that group with proteins from Arabidopsis, we confirmed that the plant sequences analysed by Harley and Saier [68] are all ALMTs providing further insights into the phylogeny of the ALMTs. With the new classification, those bacterial sequences that were grouped with the ALMTs in the PET family are now included in the same FUSC Clan as the ALMT family. Therefore, the PF11744 domain is present in bacterial proteins indicating an ancient common ancestry. Recent phylogeny on ALMT protein evolution either have a small sampling size or do not include fungi and bacterial sequences. With the development of genome-scale sequencing technologies, more information is available on the *ALMT* genes that extends this family beyond plants. This is demonstrated by combining the latest Pfam database and NCBI website and searching for the PF11744 Pfam domain (Figure 4). Among the new sequences included in the ALMT family by using the PF11744 domain are proteins of various lengths and structures. The PF11744 domain in these proteins range from 150 to 950 residues in different species and phylogenetic analysis shows approximately 30% similarity among 564 protein sequences. The conserved regions are mainly found in the trans-membrane domains at the N-terminal ends and less frequently at the C-terminal ends. Some other members only contain part of the ALMT domain and these were excluded from the analysis. We constructed a phylogenetic tree and found that ALMT-like protein sequences are present in the bacteria, Alveolata (single-celled Eukaryotes), Stramenopiles (brown algae, oomycetes), Amoebozoa, fungi, and Viridiplantae. Within the Viridiplantae, ALMTs occur in non-vascular plants (mosses), spore-forming vascular plants (club mosses) and seed-forming vascular plants which is consistent with Dreyer et al. [20] (Figure 4). The phylogenetic analysis shows that the ALMT family is far more ancient than the plant kingdom but appears to be absent from animal genomes.

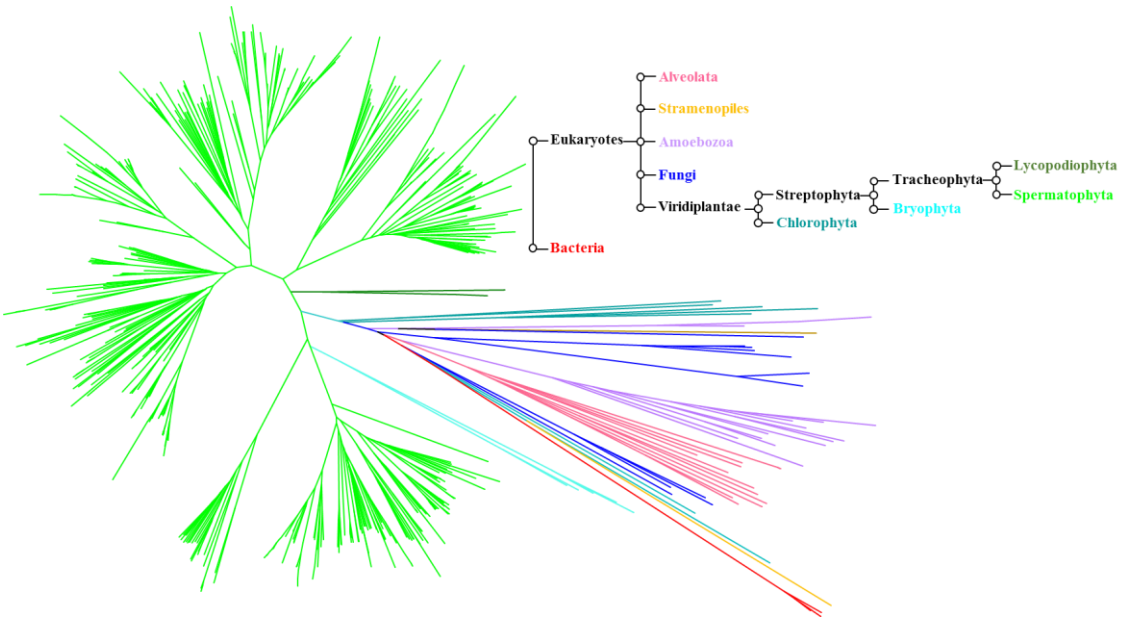

Figure 4. Unrooted phylogenetic tree of the ALMT family. The phylogenetic tree was constructed using the PF11744 domain sequences of ALMT proteins. These were obtained from the Pfam website [66]. ALMT sequences were aligned with ClustalW in the MEGA 5.0 software [69] phylogenetic tree was constructed using the neighbour-joining method. Each line represents an individual genotype. Species are represented by various colours with 5 bacteria, 2 Alveolata, 3 Stramenopiles, 4 Amoebozoa, 19 Fungi, 3 Chlorophyta, 1 Bryophyta, 1 Lycopodiophyta and 53 species of Spermatophyta.

4.2. Secondary Structure

Most algorithms for secondary structure predict that plant members of the ALMT protein family have five to seven transmembrane regions in the N-terminal half and a long hydrophilic "tail" in the C-terminal half [19]. However even for the same protein sequence different programmes can predict different transmembrane arrangements. The first experimental examination of the topology of an ALMT protein was performed on TaALMT1. In that immunocytochemical approach antibodies were generated to target different regions of the TaALMT1 protein. It was concluded that both the C-terminal and N-terminal tails were orientated extracellularly [70]. More recent studies have questioned this structure and instead have suggested that the C-terminal end is oriented toward the intracellular space [20,71–74]. Secondary structures of ALMT proteins with the ALMT domain (PF11744) vary. Most of the ALMT proteins contain five to six transmembrane regions (TMRs). Although some of these proteins only contain a part of the PF11744 domain, they still have the typical structure of transmembrane regions in the N-terminal half of the protein (three to seven transmembrane regions) and a hydrophilic C-terminal 'tail'. Within this group there are various protein arrangements where some have a single PF11744 (ALMT) domain while others contain more than one PF11744 domain. For example, a grape ALMT protein comprising of 1070 amino acid residues has a repetitive structure with two almost complete ALMT domains with six transmembrane regions in each domain. Other examples include the S3 and S4 structures which contain two or three incomplete ALMT domains all connected to each other (Figure 5).

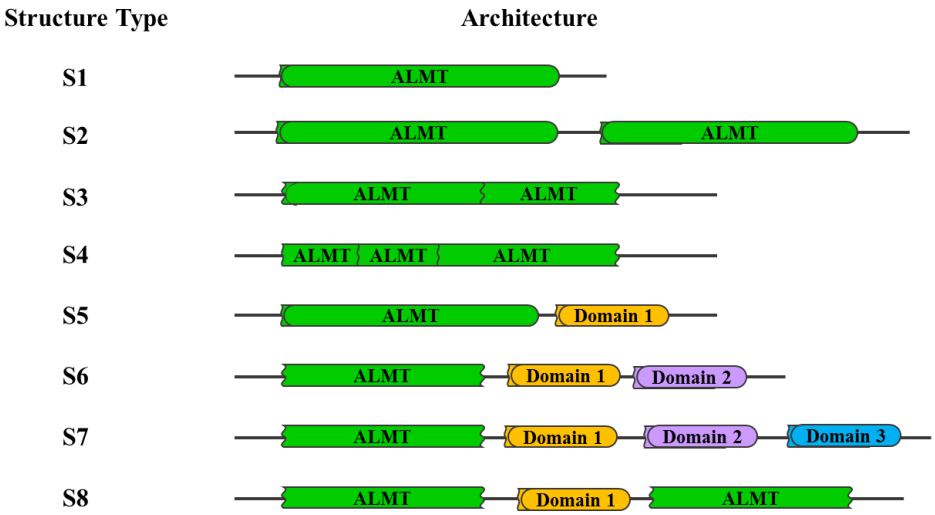

Figure 5. Structures of some members of the ALMT protein family. Members of the ALMT family of proteins may contain one or more PF11744 domains. S1 architecture is the simplest and most widespread structure and contains only one complete (or incomplete) ALMT domain. For other structures, the ALMT protein may contain more than one PF11744 domains with or without other domains. These domains may or may not be complete but maintain the same general structure. For instance, proteins with structures S5 to S8 contain domain(s) other than the ALMT domain and these are represented by Domains 1, 2, 3 (Note that these Domains do not represent a specific domain in each case but just indicate domains that are different from PF11744). (See http://pfam.xfam.org/family/ALMT for more details).

The S5, S6, S7 and S8 are composite structures with one or two ALMT domains combined with other domains (Figure 5). Some of those other domains also belong to the FUSC clan along with the ALMT domain (such as the FUSC_2, ArAE_2_N) or they belong to other clans with no relationship with ALMTs. These other domains include the EF hand which has a helix-loop-helix motif found in a large family of calcium-binding proteins, the glycoside hydrolase family 43 or domains with uncharacterised functions (such as TLD). The ALMT-like protein in *Perkinsus marinus* (Mackin, Owen & Collier, Levine 1978. Single-celled pathogen of oysters) has a more complex structure with two

ALMT domains, a FUSC_2 domain and a total of 24 predicted transmembrane regions among its 2287 residues. Most of the complex structures exist in species with an ancient history such as fungi, green algae and Alveolata. Based on these patterns we would predict that the more recent members of ALMT might not function as single subunits but function as dimers or multimers. For instance, the *Arabidopsis* AtALMT9 appears to forms a multimeric channel of four subunits [67]. By contrast, evidence collected with bimolecular fluorescence complementation (BiFC) indicates that *TaALMT1* functions as a homodimer (Pineros MA, Ligaba A, Kochian LV, personal communication).

4.3. The Structure-Function Relationship of ALMT Proteins

As discussed above, the ALMT family members have a conserved secondary structure with an N-terminus region containing transmembrane domains and a hydrophilic C-terminus sometimes comprising up to half of the length of the entire protein. Recent studies have started to examine aspects of the structure-function and especially the amino acid residues critical for activation of transport activity by Al^{3+} and for its permeability to anions (Figure 6). Studies using protein kinase and phosphatase inhibitors first indicated that reversible protein phosphorylation may be involved in activating malate efflux via ALMTs from wheat and Arabidopsis roots [75,76]. Whether responses to these inhibitors were caused by direct modifications to the ALMT proteins or due to upstream signalling cascades that later lead to malate efflux is unclear. By systematically modifying candidate amino acids in the TaALMT1 protein that could be involved in phosphorylation, it was concluded that S384 is an essential residue regulating TaALMT1 activity via direct protein phosphorylation and that this process precedes Al^{3+} enhancement of transport activity [77].

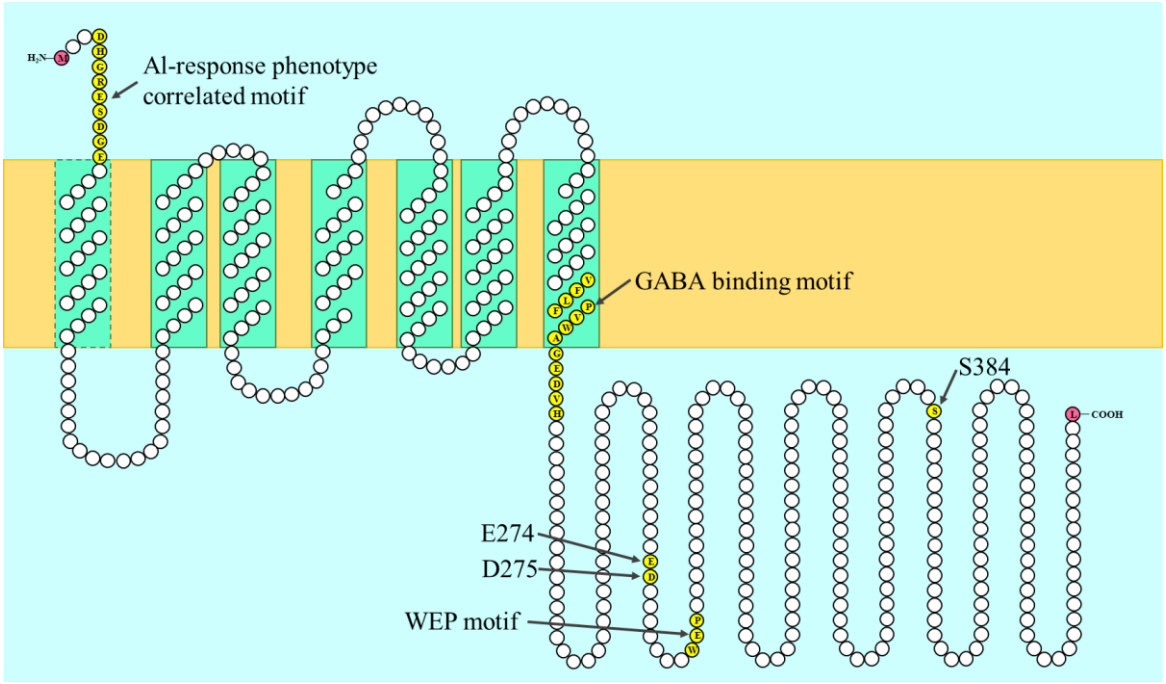

Figure 6. Hypothetical model for TaALMT1 showing residues and domains potentially important for function. TaALMT1 most likely has six transmembrane regions (green boxes with solid line border) based on a consensus of several different predictive programs for secondary structure. Another transmembrane region (green boxes with dashed line border) is also predicted by some programmes. The direction of the C-terminals ends is uncertain and will depend on the number of transmembrane regions. The "DHGRESDGE" motif within the N-terminus prior to transmembrane regions is correlated with the Al-response phenotype according to Ligaba et al. [73] and the "FLFPVWAGEDVH" motif residing near the end of the sixth transmembrane domain is the binding site for GABA. The WEP motif is highly conserved in all ALMTs. E274, D275 and S384 initially predicted to be essential for ALMT function are instead likely to be important for overall protein structural.

In oocytes expressing the S384A mutated protein, TaALMT1-dependent basal currents and the Al-enhanced currents were significantly reduced. Furthermore, the currents were insensitive to protein kinase inhibitor staurosporine and the protein kinase C (PKC) activator, phorbol 12-myristate 13-acetate (PMA). Using a similar mutational approach, Furuichi et al. [78] concluded that three acidic residues, E274Q, D275N and E284Q, located on the hydrophilic C-terminal region of TaALMT1 are important for the Al^{3+}-activated transport activity because mutations at these sites decreased the Al-dependent responses without affecting basal transport activity [78]. A comparable mutation in AtALMT1, E284Q, has the same effect [78] and the mutations E256Q in AtALMT1 and E276Q in AtALMT12 are comparable to E274Q in TaALMT1 and show the same decreases to Al-dependent transport function [72]. However subsequent studies demonstrated that those three residues in TaALMT1 (E274, D275 and E284) are highly conserved throughout the entire ALMT family, even in ALMTs that are not activated by Al. Dephosphomimetic mutants of AtALMT4 S382 showed increased channel activity and Mal^{2-} efflux. Reconstituting the active channel in almt4 mutants impaired growth and stomatal opening. Phosphomimetic mutants were electrically inactive and phenocopied the almt4 mutants and S382 can be phosphorylated by mitogen-activated protein (MAP) kinases in vitro[38]. Therefore the residues might be more important to tertiary protein structure rather than specifically involved with the activation by Al [73]. This was confirmed when Ligaba et al. [73] who showed that mutations in 43 additional acidic (negatively charged) residues of TaALMT1 also affected protein function. Indeed the residue E284 is part of a WEP fingerprint motif (Trp-Glu-Pro) which is in all ALMTs [20]. Rather than targeting specific residues to investigate the structure-function of ALMTs, other researchers have focussed on large domains of the protein. For instance, when the entire hydrophilic C-terminal region was removed from TaALMT1 or AtALMT12 the resulting proteins lost both basal and Al-dependent transport activity when expressed in *Xenopus* oocytes [78]. The Al^{3+}-dependent activity could be recovered in TaALMT1 by adding the C-terminal region from the Arabidopsis protein AtALMT1 [78]. Further experiments demonstrated that both the N- and C-domains are involved in Al-mediated enhancement of TaALMT1 transport activity. Ligaba et al. [73] proposed that a motif on the N-terminus denoted by [D,E]-[H,K,R]-x-[K,R]-[D,E]-x-x-x-[D,E] is required for this response observed in a subset of ALMTs [73]. Similar conclusions were reported by Sasaki et al. [53] who generated chimeric proteins from TaALMT1 of wheat and AtALMT1 of Arabidopsis and examined their transport function in *Xenopus* oocytes. Firstly they detected transport activity only for TaALMT1 but not for AtALMT1. However where the N- and C-terminal halves of TaALMT1 and AtALMT1 were swapped (Ta::At and At::Ta) they found transport activity whenever the N-terminal half of TaALMT1 was present [53]. Moreover, they identified a putative helical domain on the N-terminal and another on the C-terminal ends of TaALMT1 which are important for its transport activity [53].

In fact, the topology of ALMT transporters remains unclear and doubt still remains whether the N and the C-terminal ends are intracellular or extracellular [6,8,20,70,72,79]. Recently Ramesh et al. [80] showed that the transport activity of several ALMTs expressed in *Xenopus* oocytes is very sensitive to inhibition by gamma-aminobutyric acid (GABA) and muscimol, an agonist of $GABA_A$ receptor. Subsequent studies suggest that ALMT proteins may be GABA receptors in plants and underlay novel signalling pathways associated with stress responses. Although the ALMTs have a low overall sequence similarity with the $GABA_A$ receptors in animal cells, ALMTs contain a conserved motif that is similar to the GABA binding site on $GABA_A$ receptors. This intriguing result suggests that ALMTs may perform many other functions in plants which are still to be discovered [80].

5. Conclusions

Although sharing a same name of ALMT and a similar secondary structure, members from this gene family vary from each other's. Little is known about regulation mechanism and core motifs for different functions. Further studies need to be done to clarify the function and regulation of new members as well as the characterised members.

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Author Contributions: J.L. prepared the manuscript. M.Z. reviewed and revised the manuscript.

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